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Review

Sphingolipid/cholesterol regulation of neurotransmitter receptor conformation and function

Jacques Fantini [a], Francisco J. Barrantes [b,*]

[a] Centre de Recherche en Neurobiologie et Neurophysiologie de Marseille (CRN2M), University of Aix-Marseille 2 and Aix-Marseille 3, CNRS UMR 6231, INRA USC 2027, Faculté des Sciences de St. Jérôme, Laboratoire des Interactions Moléculaires et Systèmes Membranaires, Marseille, France
[b] Instituto de Investigaciones Bioquímicas de Bahía Blanca and UNESCO Chair of Biophysics and Molecular Neurobiology, Universidad Nacional del Sur-CONICET, C.C. 857, Bahía Blanca, B8000FWB Buenos Aires, Argentina

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ABSTRACT

Like all other monomeric or multimeric transmembrane proteins, receptors for neurotransmitters are surrounded by a shell of lipids which form an interfacial boundary between the protein and the bulk membrane. Among these lipids, cholesterol and sphingolipids have attracted much attention because of their well-known propensity to segregate into ordered platform domains commonly referred to as lipid rafts. In this review we present a critical analysis of the molecular mechanisms involved in the interaction of cholesterol/sphingolipids with neurotransmitter receptors, in particular acetylcholine and serotonin receptors, chosen as representative members of ligand-gated ion channels and G protein-coupled receptors. Cholesterol and sphingolipids interact with these receptors through typical binding sites located in both the transmembrane helices and the extracellular loops. By altering the conformation of the receptors ("chaperone-like" effect), these lipids can regulate neurotransmitter binding, signal transducing functions, and, in the case of multimeric receptors, subunit assembly and subsequent receptor trafficking to the cell surface. Several sphingolipids (especially gangliosides) also exhibit low/moderate affinity for neurotransmitters. We suggest that such lipids could facilitate (i) the attachment of neurotransmitters to the postsynaptic membrane and in some cases (ii) their subsequent delivery to specific protein receptors. Overall, various experimental approaches provide converging evidence that the biological functions of neurotransmitters and their receptors are highly dependent upon sphingolipids and cholesterol, which are active partners of synaptic transmission. Several decades of research have been necessary to untangle the skein of a complex network of molecular interactions between neurotransmitters, their receptors, cholesterol and sphingolipids. This sophisticated crosstalk between all four distinctive partners may allow a fine biochemical tuning of synaptic transmission.

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* Corresponding author. Tel.: +54 291 4861201; fax: +54 291 4861200.
E-mail address: rtfjb1@criba.edu.ar (F.J. Barrantes).

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1. Scope of the review

Lipids are the most abundant organic compounds found in the brain, accounting for up to 50% of its dry weight [1]. As in other mammalian tissues, brain lipids consist mainly of three major categories: glycerophospholipids, sphingolipids and cholesterol [2]. Most of these lipids are part of membrane structures. Since the original formulation of the mosaic fluid model by Singer and Nicolson [3], it has become evident that the role of membrane lipids is not just to provide a bidimensional solvent for membrane proteins: they are also involved in key biological functions linked to the spatial organization of the cells, e.g., cellular adhesion [4], toxin and pathogen attachment [5], signal transduction [6] or protein trafficking [7]. Sphingolipids and cholesterol, which coalesce and form ordered platform domains (commonly referred to as lipid "rafts") that are segregated from the bulk membrane, are privileged actors of these critical processes. The biochemical composition, biophysical properties, detergent solubility and biological functions of these lipid domains have been analyzed and discussed in several excellent reviews [8–13]. Despite these synthetic efforts, one fundamental issue warrants further consideration: proteins that are associated with lipid domains might be surrounded by a shell of typical raft lipids (sphingolipids, cholesterol) which mediates the transfer of the protein from the bulk membrane to the domain [7]. This lipid shell or annulus is analogous to the layer of solvent (water) molecules surrounding soluble proteins: in both cases, a rigid layer of tightly bound solvent molecules separates the protein from the bulk solvent. What are the biochemical mechanisms and the molecular forces involved in these lipid–protein interactions? To what extent are they specific? What is the impact of these interactions on the conformation and further on the functional activity of the proteins? Sphingolipids were discovered in the brain more than one century ago [14], but their functions in neural tissues are far from being understood [15]. Nevertheless, a growing body of data suggests that sphingolipids and/or cholesterol are indispensable partners of neurotransmitter receptor conformation, function and trafficking. The scope of this review is to bring together these data and to propose a general model accounting for this major but generally underappreciated function of domain-forming lipids. To remain concise and comprehensive, our discussion will be mainly focused on acetylcholine and serotonin receptors, i.e., two key members of the Cys-loop superfamily of ligand-gated ion channels (nicotinic acetylcholine receptor, 5-HT3 serotonin receptor) as well as representative members of G protein-coupled receptors (5-HT1, 5-HT2, 5-HT4, 5-HT5, 5-HT6 and 5-HT7 serotonin receptors).

2. Sphingolipids in neural tissues: historical findings, current status and perspectives

The history of sphingolipids begins at the end of the nineteenth century when J.L.W. Thudichum discovered a group of glycosidic substances in brain, composed of three building blocks: a fatty acid, a long chain base, and a hexose [14]. Thudichum coined the word 'sphingosine' to designate the long-chain base 'in commemoration of the many enigmas which it presented to the inquirer,' in reference to the Sphinx enigmas of Greek mythology. Early investigators identified it as di-hydroxyamino-octadecene, but the relative position of the functional groups remained uncertain until Carter and co-workers reported the correct structure in 1947 [16]. Interestingly, and contrary to a widespread extrapolation [17], the term 'sphingolipid' was not invented by Thudichum, but by Carter et al. who proposed to give the name 'sphingolipides' to all lipids derived from sphingosine [18]. In 1958, Shapiro et al. published the total synthesis of sphingosine, confirming its chemical structure as trans-D-erythro-1,3-dihydroxy-2-amino-4-octadecene (or, according to the R/S system, trans-(2*S*,3*R*)-1,3-dihydroxy-2-amino-4-octadecene [19] (Fig. 1). Subsequently it appeared that even though sphingosine is by far the main sphingoid base found in mammals, a number of other derivatives such as sphinganine (i.e., sphingosine without the trans-C4–C5 double bond) and phytosphingosine (C4–OH sphinganine) have also been detected [20,21]. Correspondingly, the definition of sphingolipids as sphingosine-derived lipids [18] has been tacitly extended to all lipids derived from sphingosine and structurally related sphingoid bases. Sphingolipids exhibit a huge heterogeneity in their structure [22]. The nature of the sphingoid base is the first level of biochemical diversity in sphingolipids. The second level is provided by the numerous fatty acids (more than 20, varying in chain length, degree of saturation, and degree of hydroxylation) that can be attached to the sphingoid base to form a ceramide (Fig. 1). The third level of diversity corresponds to the polar group linked to ceramide, whose chemical structure determines the sphingolipid classification: phosphorylcholine for sphingomyelins (SM), or a glycone moiety for glycosphingolipids (GSLs). The structures shown in Fig. 1 illustrate how distinct in shape these sphingolipids can be. Several hundreds of different carbohydrate structures have been described in GSLs, which, by combining the three levels of diversity described above, comprise *thousands* of biochemically distinct molecules [23]. GSLs can be either neutral or electrically charged at pH 7. Neural tissues contain sulfated GSLs (sulfatides) and sialic acid-containing GSLs (gangliosides) both negatively charged [1], but also several types of cationic GSLs [24] (Fig. 1). In the central and peripheral nervous system, the most abundant neutral GSL is galactosylceramide (GalCer), which accounts for as much as 24% of myelin [25]. The sulfated congener of GalCer (galactosylsulfatide, i.e., 3′-sulfoGalcer), generally referred to as sulfatide, is also present in the myelin sheath (4%). Studies with knock-out mice suggested that both GSLs play important roles in myelin function and stability [26]. Gangliosides, the sialic acid-containing GSLs, were both isolated and named by Klenk in 1942 [27]. The main gangliosides in the mammalian central nervous system are GM1, GD1a, GD1b, and GT1b, which together account for 80–90% of the total gangliosides [1]. Their concentration in the extracellular-facing monolayer of neural plasma membranes has been estimated to be 10–20% of the total membrane lipid [25]. Gangliosides have been implicated in major neural functions including brain development, neuritogenesis, memory formation, synaptic transmission, and aging

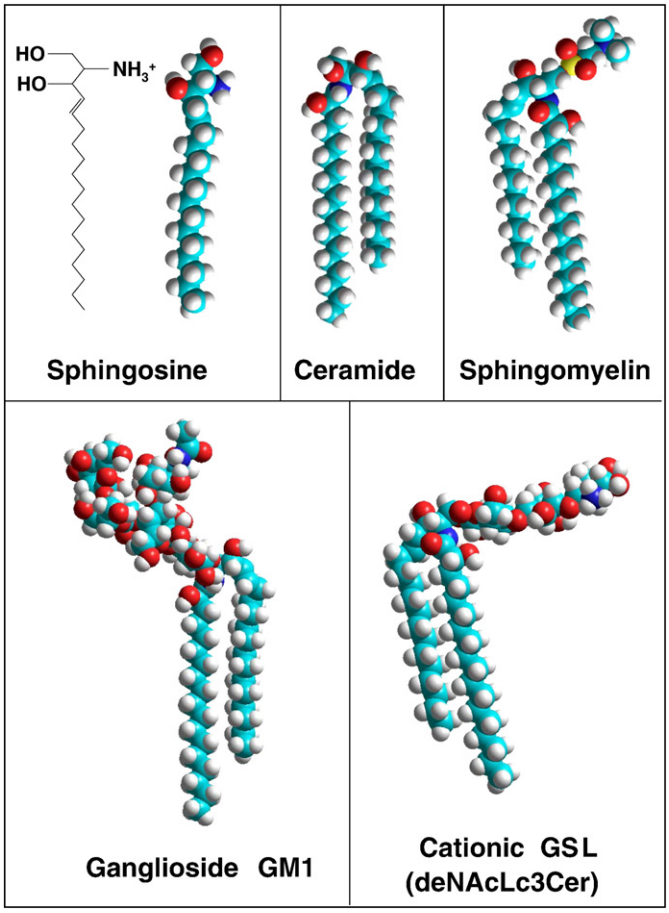

Fig. 1. Diversity of sphingolipid structures. Chemical structure of sphingosine (upper left): according to the Fisher convention, the amino group on the right side of the carbon chain (D series) and the two OH groups on the same side (erythro configuration) explain why the molecule is referred to as D-erythro-sphingosine. Yet this representation does not fit with the more realistic conformation of sphingosine shown on the right (minimum energy conformer simulated in vacuo with the Polak–Ribiere algorithm of the Hyperchem software). The other sphingolipid structures have also been obtained by molecular modelling simulations in vacuo. The ceramide contains an alpha-hydroxylated acyl chain (ceramide-HFA).

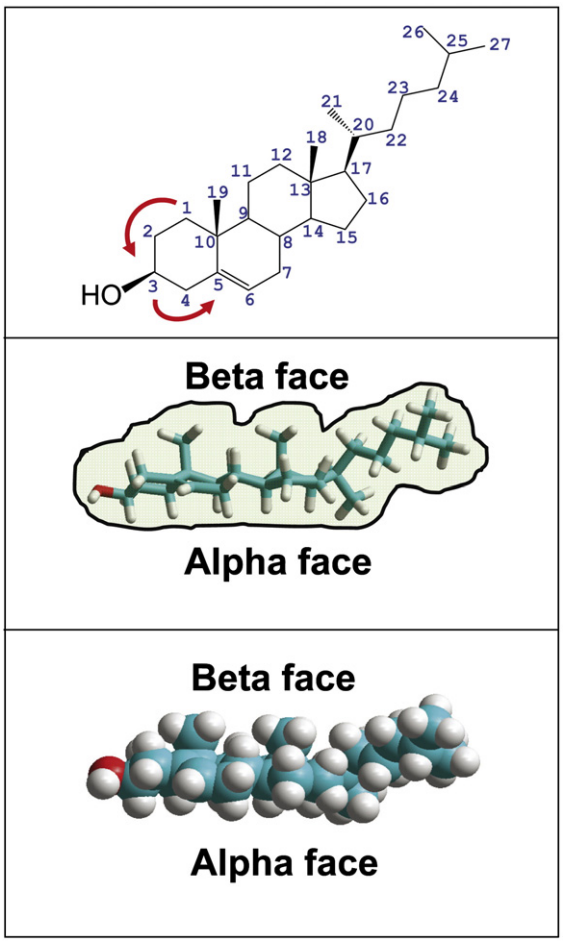

Fig. 2. Chemical structure of cholesterol with special emphasis of its bifacial geometry. (A) Structure of cholesterol with numbering of the carbon atoms. If we follow the order of carbon numeration of the first cycle, we are moving in the anti-clockwise direction which defines the beta face, according to the nomenclature proposed by I.A. Rose for cyclic compounds [35]. (B) Global shape of the cholesterol molecule, with a smooth face devoid of substituents (alpha) and a rough face (beta) with two methyl groups and the iso-octyl chain. (C) Space-filling model of cholesterol.

[28]. To conclude this brief survey of the GSL expressed in neural tissues, one should also mention the existence of cationic GSLs, such as de-*N*-acetyllactotriaosylceramide (GlcNH$_2$β1-3Galβ1-4GlcCer or de-Nacetyl-Lc3Cer) (Fig. 1). This singular GSL has been purified relatively recently from bovine brain white matter by Hakomori and co-workers [29]. These authors suggested that such cationic GSLs could affect the organizational assembly of GSLs in the membrane, which are almost all either anionic or neutral, resulting in perturbations of signal transduction. This opens an attractive and rather unexpected new avenue in brain research which warrants further experimental examination.

3. Cholesterol: the bifacial lipid

Cholesterol is the privileged lipid partner of sphingolipids in membrane microdomains. In the nervous system, cholesterol is also the main lipid component of myelin (28%) [25]. The structure of cholesterol is shown in Fig. 2. The biochemistry, physical properties and functions of cholesterol in relation to sphingolipid association and lipid domain formation have been the subject of excellent experimental studies and reviews [10,30–34], and we will therefore focus on specific structural features directly related to the scope of the present article. Perhaps the most widely ignored structural property of cholesterol is that it is a dissymmetrical molecule with two distinct faces, one smooth and the other rough due to the methyl substituents of carbons C10 and C13, and the iso-octyl chain linked to C17. According to the alpha/beta-face system numeration of ring compounds proposed by Rose et al. [35], the planar smooth face of cholesterol is referred to as the alpha face, the opposite rough side being the beta face (see legend of Fig. 2 for explanation). This topological dissymmetry allows one molecule of cholesterol to interact with two distinct membrane molecules, e.g., a sphingolipid through the alpha face [36] and a transmembrane protein through the beta face [37]. This unique biochemical feature of cholesterol will be further discussed below (see Fig. 11).

4. Neurotransmitters, sphingolipids, cholesterol and receptors: from molecular interactions to physiological regulation

4.1. Bidimensional concentration of neurotransmitters through binding to cell surface GSLs

Synaptic vesicles contain very high concentrations of neurotransmitters (up to 600 mM), thus favouring the formation of non-covalent oligomers (aggregates) as demonstrated for serotonin [38]. Upon release of the vesicles' content in the synaptic space, the neurotransmitter concentration is suddenly decreased such that monomers are spontaneously released from the aggregates. To exert its biological

effect, the neurotransmitter has to reach the postsynaptic membrane where it binds to its target receptor, a protein macromolecule. The transfer of neuromediator molecules from a three-dimensional space (the synaptic space) to a two-dimensional one (the post-synaptic membrane) results in a further increase in neurotransmitter concentration through a typical mechanism of reduction in dimensionality [39]. It has been known for many years that neurotransmitters such as serotonin bind to several gangliosides known to be present on neural cell membranes [40,41]. In fact, these GSLs had been considered for quite some time to be part of the neurotransmitter receptors, a hypothesis that was definitively abandoned once the real protein receptors were identified and characterized. Nevertheless, it is still reasonable to assume that the low affinity binding of neurotransmitters to acidic GSLs [42] is an efficient way to 'catch' neurotransmitters onto the postsynaptic membrane (i.e., a concentrating effect) and also to prevent their aggregation. This scenario has been visualized for serotonin (Fig. 3). Molecular dynamics simulations were conducted to figure out the possible effects of the ganglioside on the bioavailability of serotonin in the vicinity of the post-synaptic membrane. Our data suggested that the ganglioside could (i) destabilize serotonin aggregates and (ii) bind to monomeric serotonin with a low/moderate affinity. The neurotransmitter molecules that are released from the aggregates can either bind to a receptor in the post-synaptic membrane, or be re-captured by the pre-synaptic neuron. On the other hand, the neurotransmitter monomers that interact with the post-synaptic membrane can be routed and transferred to their high-affinity protein receptors (Fig. 3).

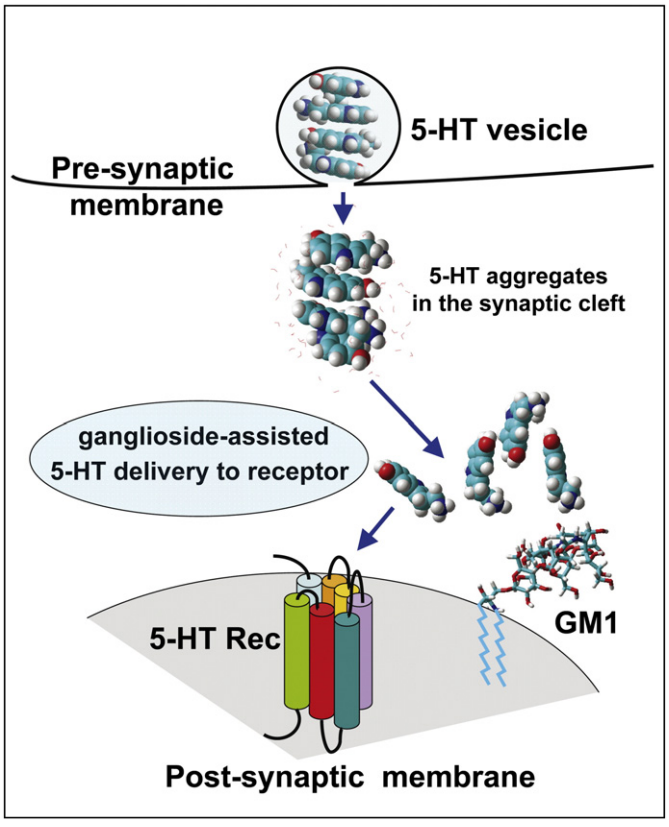

Fig. 3. Putative accessory function of ganglioside binding sites for neurotransmitters on the post-synaptic membrane. In the synaptic vesicle, the neurotransmitter concentration is high (up to 600 mM), which favours its aggregation through non-covalent interactions, as shown for serotonin (5-HT). Upon release in the synaptic cleft, some aggregates are spontaneously dislocated, whereas others are electrostatically attracted by the gangliosides (e.g., GM1) present in the post-synaptic membrane. These gangliosides have two main effects: (i) they contribute to destabilizing neurotransmitter aggregates, and (ii) they can interact with neurotransmitter monomers, through low/moderate affinity interactions. Eventually, the monomers will reach specific protein receptors in the post-synaptic membrane. This concept of a dual GSL/receptor system for neurotransmitters in the post-synaptic membrane is similar to the double receptor model developed by C. Montecucco for the binding of neurotoxins to neuronal membranes [47,48].

In addition to serotonin [40–43], both GABA and glutamate have been reported to interact with sulfatide as well as various phospholipids including sphingomyelin [44]. Peptidic neurotransmitters such as leucine enkephalin, alpha-MSH and substance P have also been shown to interact with GSLs [45,46], suggesting that binding to cell surface sphingolipids might be a general property of neurotransmitter molecules. As pointed out by Montecucco in his double-receptor model [47,48], a similar strategy based on the cooperation of gangliosides and protein receptors seems to be used by neurotoxins such as the botulinum toxin to achieve optimal binding to neural cells.

4.2. Regulation of receptor function by gangliosides

In aqueous solution, gangliosides form micelles which, upon incubation with cell or tissue cultures, can transfer these charged GSLs to the plasma membrane of recipient cells [49]. Several researchers have taken advantage of this property to appreciate ganglioside action in the nervous system by (i) adding gangliosides to neurons in culture, or (ii) treating animals during neuronal regeneration [50]. This made it possible to demonstrate that ganglioside GM1 enhances neurite outgrowth in PC-12 cells, through NGF and integrin-dependent mechanisms [51,52]. Overall, gangliosides were found to increase the rate and extent of sprouting of regenerating axons and enhance neuronal differentiation and sprouting in vitro and in vivo [50]. Direct effects of gangliosides on receptor function have also been characterized. In particular, gangliosides GM1 and GQ1b have a marked stimulatory effect on the functional coupling between adenylate cyclase and serotonin receptors [53]. Similarly, GM1 treatment of cultured neurons was reported to induce an increased efficacy of excitatory opioid receptor-mediated Gs activation [54]. This has led to the striking conclusion that GM1 could contribute to opioid tolerance and dependence by regulating the excitatory signalling of G-coupled opioid receptors [55]. Indeed, decreasing neuronal levels of GM1 with a neuraminidase inhibitor blocked morphine's hyperalgesic effects in mice. Overall, these data demonstrate that the functional activity of representative neurotransmitter receptors can be regulated by exogenous gangliosides and, in some cases, by pharmacological alteration of endogenous ganglioside content. Does this mean that endogenous gangliosides are intimately associated with neurotransmitter receptors and that these membrane sphingolipids can affect their signalling properties? Such a simple interpretation of the data obtained with exogenously added ganglioside is not always possible. Indeed, three modes of interaction between ganglioside micelles and target cells have been described: (i) weak association with the plasma membrane (readily washable with saline), (ii) efficient adsorption on membrane proteins (removed by proteolytic digestion), and (iii) stable insertion within the outer leaflet of the membrane (protease-resistant) [49]. As these distinct modes of association may not be mutually exclusive, this renders difficult the interpretation of the data obtained with micellar ganglioside solutions. In particular, it may not be easy to distinguish the effects resulting from an interaction in *cis* (ganglioside inserted in the plasma membrane of the responding cell) from an interaction in *trans* (ganglioside bound to but not inserted in the membrane of the target cell). Ideally, a combination of techniques including pharmacological modulation of sphingolipid metabolism [56,57] and/or chemically induced alteration of lipid domain integrity and functions [58,59] should be used in parallel to treatment with ganglioside micelles.

4.3. Physical association of GSLs with membrane receptors

Despite the potential caveats discussed above, the concept of a functional association between GSLs and membrane proteins has

received careful consideration [60]. For instance, it is now well established that selected GSLs bind to the receptors for tyrothropin [61], vitronectin [62], insulin [63], EGF [64], PDGF [65], as well as other growth factors involved in brain tumours [66]. A GSL-mediated regulation of a neurotransmitter receptor through a molecular interaction with ganglioside GM1 has been reported for the delta-type opioid receptor [67,68]. This is in line with the studies showing that ganglioside GM1 plays a crucial role in regulating excitatory opioid receptor function, with a marked impact on opioid dependence, tolerance, and supersensitivity [54,55]. This receptor belongs to the family of G protein-coupled receptors, integral membrane proteins with seven membrane-spanning domains. G protein-coupled receptors respond to a wide range of stimuli (light, odour, taste) and chemical agents (neurotransmitters, lipid analogues). Upon ligand binding, these receptors undergo conformational changes which trigger the functional coupling of an intracellular domain of the receptor to a heterotrimeric G protein. The data obtained with CHO cells transfected with the delta opioid receptor suggested that GM1 induces a conformational change of the opioid receptor from a form coupled primarily to Gi (inhibiting cAMP production) to one also capable of interacting with Gs (stimulating cAMP production) [68]. This indicates that the GSLs that bind to such receptors can induce important alterations in their activity through conformational effects.

In some instances, the affinity of the receptor for the GSLs can be high enough to resist detergent solubilization and purification procedures [62,69]. When this is the case, it is noteworthy that only one GSL species is carried along with the receptor during the purification process. Such a high selectivity of the co-purified GSL argues against a 'reminiscence' of the biochemical composition of the membrane domain from which the receptor has been extracted. Instead, these data suggested that GSLs can physically interact with a subset of membrane receptors, which, in total agreement with the lipid shell concept [7], segregate into sphingolipid/cholesterol-enriched microdomains of the plasma membrane. Most of the data available on sphingolipid-mediated receptor regulation concern GSLs and chiefly gangliosides. Yet, as we will see for the nicotinic acetylcholine receptor, sphingomyelin can also be a very efficient modulator of receptor assembly and trafficking [70].

4.4. Effects of cholesterol on receptor function

That cholesterol could, like sphingolipids, regulate the function of membrane receptors, has been recognized for several years [71]. Cholesterol has been shown to modulate membrane receptor function through two main mechanisms: direct interaction with the receptor or broad effect on the biophysical properties of the membrane lipid bilayer [72,73]. In the nervous system, several G protein-coupled receptors have been particularly well studied for their functional dependence on cholesterol, e.g., rhodopsin, the photoreceptor of the rod cell, the receptors for the neuropeptides oxytocin and cholecystokinin, and the receptors of the neurotransmitters acetylcholine and serotonin. Rhodopsin is a classical G protein-coupled receptor, activated by light. High cholesterol concentrations were found to impair the functional light-induced coupling of rhodopsin to transducin (the rhodopsin-coupled G protein) in reconstituted bilayer membranes [74]. This effect was attributed to cholesterol modulation of the properties of the membrane bilayer, resulting in a decrease in the free volume available for molecular motion in the hydrophobic core of the bilayer [74]. In addition to this indirect effect, Albert et al. [75] were able to demonstrate a specific interaction between cholesterol and rhodopsin. Taken together, these data raised the intriguing possibility that the regulatory effects of cholesterol on rhodopsin activity could involve direct and indirect mechanisms. A direct interaction with cholesterol has also been demonstrated for the G protein-coupled receptor for oxytocin, a neurohypophyseal nonapeptide [76,77]. In contrast, the human cholecystokinin receptors subtype B did not bind cholesterol but were indirectly affected by the changes in physical properties associated with modulations of membrane cholesterol content [78]. These receptors have been involved in important functions of the nervous system including analgesia [79] and exploratory behavior [80]. Again, these data showed that cholesterol could alter brain receptors by two distinct mechanisms, i.e. physical interaction with the receptor and non-specific changes of biophysical membrane properties [72]. The different mechanisms by which cholesterol could interact with G protein-coupled receptors have been recently reviewed [81]. In view of the scope of the present work, we will limit our discussion to those involved in the regulation of nicotinic acetylcholine and serotonin receptors by membrane cholesterol.

5. Serotonin receptors

5.1. The serotonin molecule: structural features and binding to gangliosides

The chemical structure of serotonin (5-hydroxytryptamine, 5-HT) [82] is shown in Fig. 4A. It is a nitrogen-containing aromatic cycle (indole) with a hydroxy substituent in the 5-position on the ring (5-OH group) and an ethylamine chain with is positively charged at pH 7. The aromatic structure is primarily responsible for the self-aggregation properties of serotonin in synaptic vesicles [38]. Molecular modelling simulations of serotonin aggregates in water showed a stacked geometry of interaction (Fig. 4B), which is remarkably similar to other stacking interactions between aromatic compounds (e.g., adenine) [83]. The presence of a positive charge on the ethylamine chain, together with the aromatic cycle, confer on serotonin the ability to interact with gangliosides [40–43], as shown in Fig. 4C for GM1. In this molecular model, the CH3 of the *N*-acetyl group borne by the sialic acid of GM1 is oriented towards the aromatic ring of serotonin, consistent with the establishment of a CH–Pi interaction [84]. Moreover, the positive charge of the amino group of serotonin interacts with the negative charge of the sialic acid residue of GM1. Overall, the combination of an electrostatic plus a CH–Pi interaction is consistent with a low to moderate affinity for the serotonin/GM1 complex. Such molecular interactions between serotonin monomers and GM1 might take place following the release of the neurotransmitter from synaptic vesicles, as described in Fig. 3.

Despite its simple structure, serotonin plays a significant role in a number of fundamental biological processes such as the regulation of mood, stress, sleep, human sexuality, appetite, and metabolism [85]. A first level of explanation for such pleiotropic effect would be the occurrence of multiple serotonin conformers able to stimulate distinct receptors. The flexibility of the ethylamine chain and the multiple possible orientations of the 5-OH group, which can generate a wide range of conformations, strongly support this view [86]. One can hypothesize that upon binding to post-synaptic GSLs, specific conformations of serotonin could be favoured on the basis of GSL structure (Fig. 4C). If this assumption is correct, GSLs could function as moulds for serotonin so that this initial step of binding to the post-synaptic membrane would become essential for the pre-selection of serotonin receptor subsets. This would help serotonin to find its way in the intricate network constituted by its numerous membrane receptors.

5.2. Serotonin receptors: an endless complexity

Seven distinct families of serotonin receptors (numbered 5-HT1 to 5-HT7) have been identified so far, and subpopulations have been described for several of these [87]. With the exception of the 5-HT3 receptor, a ligand-gated Na^+ and K^+ cation channel, all other serotonin receptors are coupled to G proteins. The 5-HT1 and 5-HT5A subtypes are coupled to Gi (decreasing cAMP levels), whereas the 5-HT4, 5-HT6 and 5-HT7 are coupled to Gs (increasing cAMP

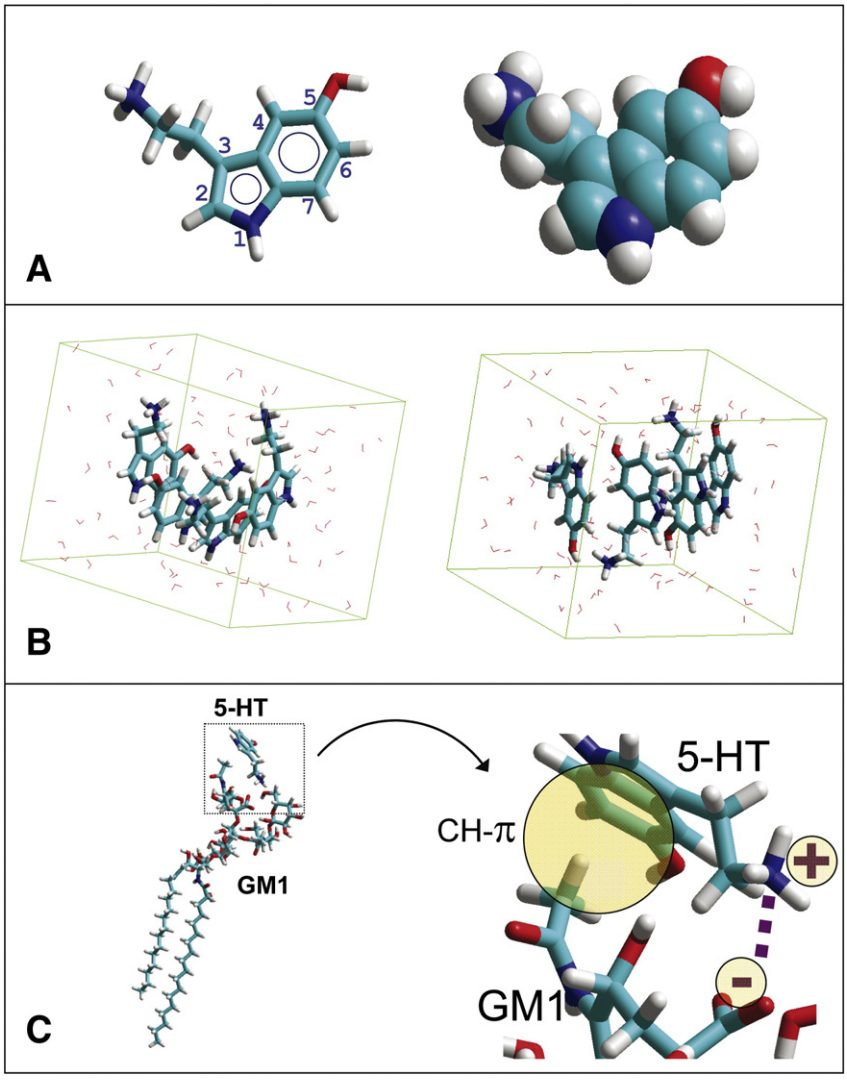

Fig. 4. Chemical structure of serotonin: from monomers to aggregates. (A) Chemical structure of serotonin. Serotonin is represented as a tube model with carbon numbering (left panel) or as a spacefill model (right panel). (B) Serotonin aggregates in water. Four molecules of serotonin have been placed in a periodic box with 185 water molecules. Following geometry optimization with the Polak–Ribiere algorithm, molecular dynamics simulations (MM+ force field) were conducted for 1 ps. Two distinct orientations of the periodic box are shown, both showing the well-organized stacking geometry of the serotonin molecules. (C) Binding of serotonin to GM1: importance of electrostatic and CH–Pi interactions. A molecular model of serotonin bound to GM1 has been obtained in vacuo with the Hyperchem software. The molecular forces involved in the complex are (i) the CH–Pi interaction between the CH_3 of the *N*-acetyl group of the sialic acid residue in GM1 and the aromatic cycle of serotonin (yellow disc), and (ii) the electrostatic interaction between the protonated amino group of serotonin and the anionic carboxylate of the sialic acid (purple dotted line).

levels). The last subtype, 5-HT2, is coupled to Gq (increasing inositol triphosphate and diacylglycerol levels). The complexity of the system is further expanded by posttranslational modifications, such as alternate splicing and RNA editing, oligomerisation, and crosstalk within and possibly between receptor families [88]. The ultimate stabilization, functional coupling and crosstalk between serotonin receptors in the plasma membrane offer multiple regulatory possibilities for cholesterol and sphingolipids, as discussed below.

5.3. G protein-coupled serotonin receptors: structural and functional links with sphingolipids and cholesterol

A first approach to evaluating the functional impact of sphingolipids and/or cholesterol on G protein-coupled serotonin receptors is to study their association with lipid domains. It is somewhat surprising that this important issue has not received much attention. In January 2009, the PubMed query 'serotonin receptor lipid raft' retrieved only six articles, compared with 81 for 'neurotransmitter receptor lipid raft,' 799 for 'receptor lipid raft' and 2270 for 'lipid raft.' The following G protein-coupled serotonin receptors have been unambiguously found in detergent-resistant ("raft") fractions: 5-HT1A [89], 5-HT2A [90], and 5-HT7 [91]. The double palmitoylation of 5-HT4 suggests its recruitment in lipid domains [92], as demonstrated later for 5-HT1A [89]. 5-HT6 interacts with Fyn [93], a member of the Src family of non-receptor protein-tyrosine kinase known to be enriched in lipid domains [94]. In addition to these receptors, the serotonin transporter (SERT) has also been detected in lipid domain fractions of pre-synaptic membranes [95]. This integral membrane protein plays a prominent role in the regulation of serotoninergic neurotransmission and is a molecular target for multiple antidepressants. The simultaneous presence of serotonin with G protein-coupled receptors and of serotonin with SERT, in sphingolipid/cholesterol plasma membrane microdomains, indicates that lipid domains behave as functional platforms able to coordinate several serotonin functions in both the pre- and post-synaptic membranes.

The function of several of G protein-coupled receptors has been shown to be regulated by lipid domains [96]. All the available data suggest that this is also the case for G protein-coupled serotonin

receptors. Indeed, disruption of lipid domain integrity through cholesterol depletion affected both ligand binding activity and G protein coupling of 5-HT1A receptors in bovine hippocampal membranes [97]. Mutations of the palmitoylation sites (which control the association with lipid domains) in mouse 5-HT1A receptors abolished the functional coupling of these receptors with G proteins [89,98]. Palmitoylation also plays an important role in modulating mouse 5-HT4A receptor functions [92]. Reduction in sphingomyelin and GSLs levels upon treatment with metabolic inhibitors resulted in decreased serotonin binding to 5-HT7 receptors in transfected HeLa cells [91]. Caveolin-1, a cholesterol-binding protein constitutive of a subtype of lipid domains (referred to as caveolae), interacts with 5-HT2A receptors and facilitates the functional coupling between 5-HT2A and Gq proteins [99]. Moreover, caveolin-1 silencing with siRNA caused significant reductions of serotonin binding to 5-HT7 receptors [100]. Taken together, these data suggest that the domain localization of several (if not all) G protein-coupled serotonin receptors is critical for ligand binding and/or receptor-mediated signaling. A plausible interpretation of these findings is that domain lipids (cholesterol, sphingomyelin, GSLs) can bind to these receptors and affect their conformation, thereby regulating both serotonin binding and G protein coupling. Despite the scarcity of high-resolution three-dimensional structures for G protein-coupled receptors [87,101], the available structural data are highly informative and may make it possible to determine whether such regulations are consistent with the topology of G protein-coupled serotonin receptors.

G protein-coupled receptors consist of a bundle of seven transmembrane alpha helices, with the amino terminus and three interhelical loops on the extracellular side and three more loops, together with the carboxyl terminus exposed to the cytoplasm [101]. The ligand binding site of serotonin receptors is located within the membrane, so that the amino acid residues involved in the interaction with serotonin belong to transmembrane helices [102]. This is of primary importance because such membrane-embedded binding sites might be accessible only to molecules (serotonin and/or agonists) that accumulate in the surrounding lipids, as recently studied by nuclear magnetic resonance spectroscopy [103]. This further underscores the crucial role of lipid domains as primary targets for serotonin in the post-synaptic membrane. Once bound to the receptor, serotonin induces a conformational change which propagates through the transmembrane domains, and eventually reaches the second and third intracellular (cytoplasmic) domains which bind to and activate the G proteins. A conserved motif of three amino acid residues, referred to as the DRY motif (Asp-Arg-Tyr), located at the bottom of the third transmembrane domain, has been shown to play a key role in the switch that activates G proteins upon serotonin binding to the receptor [102]. Overall, these data suggest that the regions of the 5-HT1A receptor encompassing the second and third transmembrane domains and the second intracellular loop form a coordinated unit which includes important residues involved in serotonin binding (Asp82 and Asp116) and in signal transduction (Asp133, Arg134, Tyr135) (Fig. 5). The next issue to resolve is whether this regulatory cassette contains specific binding sites for cholesterol and/or sphingolipids, which could account for the regulatory activity of these lipids on receptor function.

5.4. Cholesterol binding sites on G protein-coupled serotonin receptors

Since membrane cholesterol is required for the function of several G protein-coupled receptors [72], it was logical to look for specific cholesterol binding sites in serotonin receptors belonging to this family. Recently, Hanson et al. have identified a cholesterol binding pocket in the crystal structure of the β2-adrenergic receptor [104]. This consensus cholesterol motif is highly conserved among G protein-coupled receptors. Thus, it was possible to delineate a cholesterol binding domain in the 5-HT1A receptor by homology

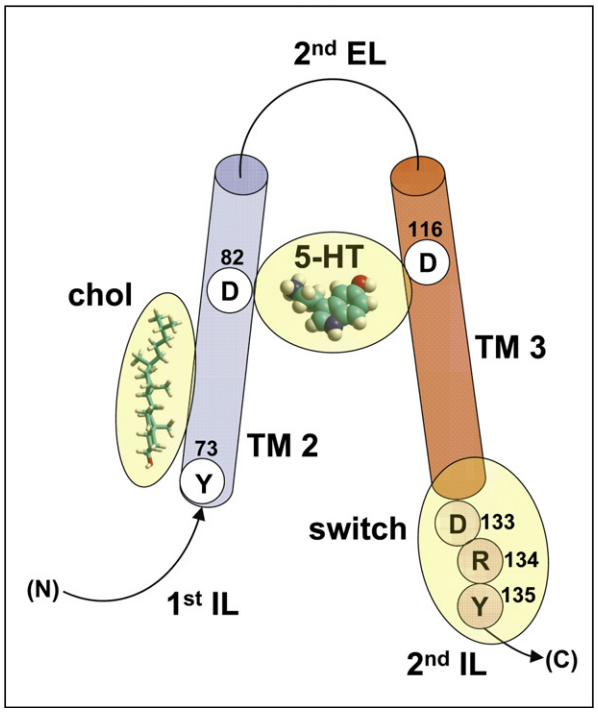

Fig. 5. A regulatory cassette in the 5-HT1A receptor: location of serotonin and cholesterol binding sites in the vicinity of the switch for G protein activation. The regulatory unit includes: (i) the second and third transmembrane helices of 5-HT1A (TM2 and TM3), which are separated by the second extracellular domain (2nd EL), (ii) the serotonin binding pocket involving amino acid residues D82 in TM2 and D116 in TM3, (iii) the DRY triad (D133–R134–Y135) in the second intracellular loop (2nd IL) at the bottom of TM3 which functions as a switch for protein G activation upon serotonin binding, and (iv) the cholesterol binding motif involving residue Y73 in TM2. The first intracellular loop (1st IL) and the orientation of the peptidic chain of the receptor (N-ter to C-ter) are indicated.

with the amino acid sequence of the β2-adrenergic receptor [81]. The cholesterol binding motif identified in 5-HT1A by these means includes Tyr73 in the second transmembrane helix (Fig. 5) and Ile157 and Trp161 in the fourth one. This topology is consistent with a regulation of serotonin binding through specific cholesterol–receptor interactions. In particular, it is highly significant that the same transmembrane domain (TM2) contains amino acid residues that are critical for interactions with both cholesterol (Tyr73) and serotonin (Asp82). This further strengthens our hypothesis that the regions of the 5-HT1A receptor encompassing the second and third transmembrane domains, as well as the second intracellular domain, form a regulatory cassette allowing the coordinated control of the receptor by serotonin and by membrane lipids (Fig. 5).

Since cholesterol might regulate the function of most G protein-coupled serotonin receptors, we searched for a similar cholesterol binding pocket in the other serotonin receptors (except for 5-HT3 which is not coupled to G proteins and has a distinct membrane topology). As shown in the amino acid alignments of Fig. 6, the cholesterol binding motif is well conserved among the different serotonin receptors coupled to G proteins. Indeed, an aromatic residue (Tyr or Phe) at the position equivalent to 73 (for 5-HT1A) is found in all the receptors studied, except for 5-HT5A (Asn). Moreover, the Trp residue at position 161 is fully conserved, whereas Ile157 is present in more than 80% of the sequences. Despite some minor variations in the region of the motif, our data suggest that all these receptors share a specific, non-annular binding site for cholesterol, as previously shown for 5-HT1A [81]. Most importantly, these findings give a biochemical explanation for the regulatory effects of cholesterol on the activity of 5-HT1A and related G protein-coupled protein receptors. Mutagenesis studies focused on the amino acid residues of this conserved cholesterol binding site will help to validate this attractive model.

Human sequence	Cholesterol binding motif
5-HT1A	ANYLIGSLAV…TPRRAAALISLTWLI
5-HT1B	ANYLIASLAV…TPKRAAVMIALVWVF
5-HT1D	ANYLIGSLAT…TAGHAATMIAIVWAI
5-HT1E	ANYLICSLAV…TAKRAALMILTVWTI
5-HT1F	ANYLICSLAV…TPKHAGIMITIVWII
5-HT2A	TNYFLMSLAI…SRTKAFLKIIAVWTI
5-HT2B	TNYFLMSLAV…SRATAFIKITVVWLI
5-HT2C	TNYFLMSLAI…SRTKAIMKIAIVWAI
5-HT4	TNYFIVSLAF…TPLRIALMLGGCWVI
5-HT5A	PHNLVASMAV…RKCVSNVMIALTWAL
5-HT6	SNFFLVSLFT…TPLRALALVLGAWSL
5-HT7	SNYLIVSLAL…NGKCMAKMILSVWLL
Consensus	:**:::S**:…:::.:+:.:*..+W.+

Fig. 6. Alignment of the putative cholesterol binding domain in the family of G protein-coupled serotonin receptors. The cholesterol binding motif has been characterized by Paila et al. in the 5-HT1A amino acid sequence in various animal species [81], by homology with the cholesterol consensus motif identified in the structure of the β2-adrenergic receptor [104]. The multiple alignments were performed with ClustalW, using the 5-HT1A sequence as reference. The characters in the consensus sequence correspond to the frequency of the amino acid at the indicated position: "." >20%, ":" >40%, "+" > 60%, "*" >80%, and the amino acid letter if 100%.

Given that sphingolipids also regulate the activity of G-protein coupled serotonin receptors [92,100], it would be interesting to look for potential sphingolipid binding sites around the second and third transmembrane domains of these receptors, for instance in the second extracellular domain.

5.5. The 5-HT3 receptor: an ionotropic ligand-gated ion channel

The 5-HT3 receptor is the only serotonin receptor subtype whose activity is not mediated via G proteins. Instead it is an ionotropic ligand-gated ion channel (LGIC) belonging to the Cys-loop superfamily of LGICs, that also includes the nicotinic acetylcholine receptor (AChR), the anion-selective GABA type A ($GABA_A$) receptor, and the glycine receptor [105]. The LGIC superfamily comprises several families of evolutionarily related neurotransmitter receptor proteins coded by a few hundreds of genes so far identified. LGICs mediate excitatory and inhibitory chemical transmission.

The common structure of the receptors belonging to the LGIC superfamily is a regular arrangement of five pseudo-symmetrically subunits surrounding a central ion-conducting pore Each subunit is composed of an extracellular domain, which contains the ligand binding domain, a transmembrane region made up of four membrane-spanning α-helices (M1–M4) and an intracellular domain [87]. The binding site is formed at the interface of two adjacent subunits by the convergence of three loops (A–C) from one subunit and three β-strands (D–F) from the adjacent (or complementary) subunit. The M2 helix from each subunit lines the pore and contains regions responsible for channel gating and ion selectivity. In the case of the ionotropic 5-HT3 receptor, this pore is predominantly sodium- and potassium-selective, and its opening results in a rapidly activating and then desensitising inward current [106]. In both stably transfected human embryonic and neuroblastoma cells, the 5-HT3A receptor was detected in lipid domains [107]. Thus the functionality of this type of 5-HT3 receptor, like most other serotonin receptors, seems to depend upon specific interactions with surrounding raft lipids. Particularly interesting is the finding that various antidepressant and antipsychotic drug co-localize with this receptor in lipid microdomains [107]. This further strengthens the idea that raft lipids (especially GSLs) are accessory binding sites for the natural and/or synthetic ligands of numerous neurotransmitter receptors (Fig. 3). It is not known whether cholesterol and/or sphingolipids can directly bind to and/or regulate the activity of the 5-HT3 ligand-gated channel. However, given the structural homology between 5-HT3 receptor and the AChR, this would be far from illogical (see below).

6. Nicotinic acetylcholine receptors

6.1. Overall structure and lipid contacts of the multimeric protein

Several genes coding for AChR subunits have been characterized in central and peripheral nervous systems. They exhibit amino acid sequence homology and presumably higher-order structural motifs. Within the LGIC superfamily, the AChR and the 5-HT3 receptor comprise two families of cation-selective channels, whereas glycine and gamma aminobutyric acid type A ($GABA_A$) receptors are anion-selective channels. Members of this superfamily are also known as Cys-loop receptors because in their amino-terminal all their subunits contain extracellular halves of a pair of disulphide-bonded cysteines separated by only 13 residues. Like several other transmembrane (TM) proteins of the large ligand-gated ion channel superfamily, the AChR exposes about half of its mass to the extracellular space, about one third lies in the intracellular compartment, and each AChR subunit contributes with four hydrophobic segments, 20–30 amino acids in length, the M1–M4 membrane-spanning or TM segments (Fig. 7). Of these, the M2 segment from each subunit contributes structurally to the formation of the ion channel proper. M4 is the segment most exposed to the bilayer lipid. M1 and M3 effectively incorporate membrane-partitioning photoactivatable probes and are also exposed, albeit less than M4, to the lipid phase. We have proposed that the extensive interface between the protein and lipid moieties, comprising both the lipid-exposed TM portions of the AChR protein and the AChR-vicinal lipid [108,109], constitutes a *functional* domain of the AChR. From a structural point of view, the lipid moiety of this interface constitutes the lipid belt ("shell," "annulus," "boundary," "AChR-vicinal") region, that is the lipid moiety in the immediate perimeter of the AChR protein, discovered using electron spin resonance (ESR)

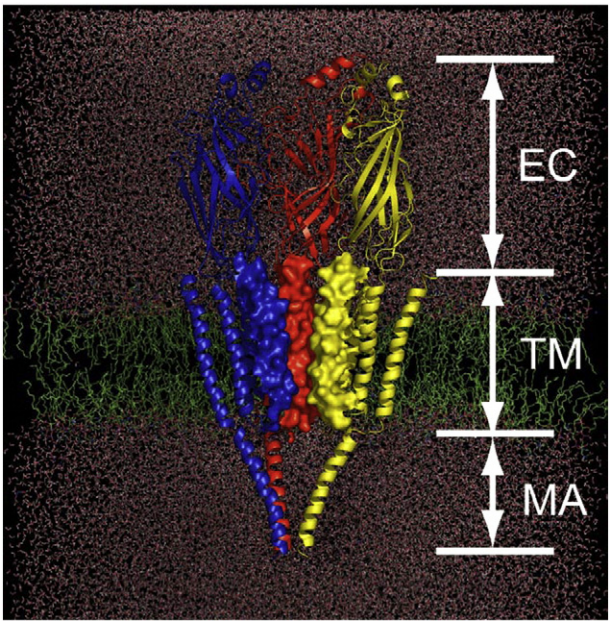

Fig. 7. The extracellular (EC), transmembrane (TM) and MA domains of the AChR are shown in a lipid bilayer. The 3D structure is based on cryoelectron microscopy data of the AChR at 4 Å resolution [131] and served as the starting conditions in a recent molecular dynamics study [191]. The receptor protein is embedded in a DPPC lipid bilayer (phospholipid acyl chains shown in green) and water molecules (red). Only three subunits (color-coded in red, blue and yellow) are displayed for clarity. The innermost ring of M2 helices is rendered with electrostatic potential surface to highlight the pore shape [191].

techniques [110] and further characterized subsequently in terms of lipid selectivity and stoichiometry [111–116]. Fig. 8 depicts a schematic representation of the AChR macromolecule in the lipid bilayer, highlighting (in red) the innermost shell of lipid molecules constituting the annular region.

Early studies from the group of McNamee [117,118] showed that the overall phase state of the membrane was also important for receptor function: the capacity of reconstituted AChR to translocate ions *in vitro* was found to be sensitive to the bulk physical properties of the host membrane. The above-mentioned physical studies on AChR-rich membranes [110,116,119] made apparent the occurrence of two distinct signals in ESR spectra of native and reconstituted membranes containing the receptor protein at relatively high or low concentrations: one signal corresponded to the bulk membrane lipid and the other was interpreted as stemming from the protein-immobilized lipid. These direct interactions between protein and lipid moieties were observed with fatty acids, phospholipids and sterols in the *native* membrane environment. Rousselet et al. [119] found immobilization with fatty acids but not with phospholipids. Ellena et al. [114] confirmed the earlier findings using reconstituted AChR. This series of studies from different laboratories demonstrated that shell or annular protein-vicinal lipids are relatively immobile with respect to the rest of the membrane lipids and pointed to the existence of phase lateral heterogeneity in membrane lipids much earlier than the concept of "rafts" came into use.

Functional studies contributed to understanding the role of lipids in AChR ion permeability. The need to include sterols and certain phospholipids to preserve this property of the AChR in reconstituted systems was subsequently demonstrated [120]. The relative contributions of phospholipid and sterol were established in various studies *in vitro* [121–123] and the minimal number of lipid molecules (~45) per AChR was ascertained in ESR experiments [114,124]. AChR-vicinal lipids appeared to be an inherently relevant environmental feature of the AChR native membrane, but the nature of the interaction between protein and lipid moieties was still obscure, as were the possible functional implications proposed in early work [110].

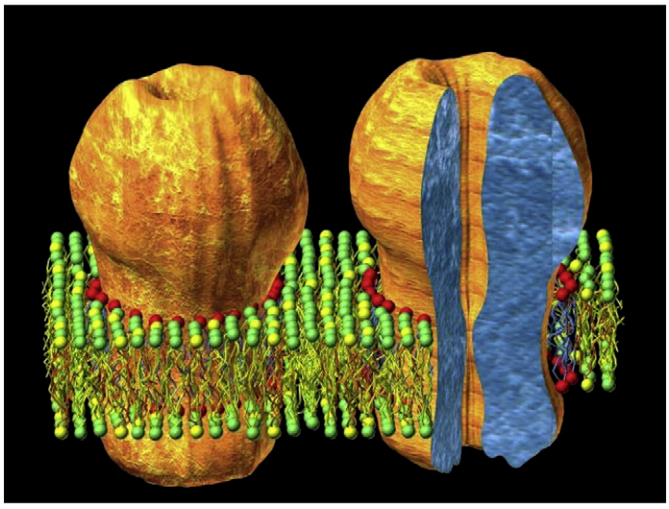

Fig. 8. The AChR and its lipid domain. The bulk lipid region is depicted as green and yellow phospholipid molecules. The red spheres immediately adjacent to the AChR molecules represent the polar head regions of the phospholipids in the first-shell ("belt layer," "annular") region [78,110,150,156]. Unlike the vast majority of individual proteins in cell-surface membranes ("iceberg-like embedded proteins in a sea of lipid"), the AChR molecules form tightly packed two-dimensional arrays in the postsynaptic membrane, in which lipid molecules fill in the interstices. Only three to four phospholipid shells of bulk lipid separate the protein-vicinal first shell from an homologous, adjacent one surrounding a neighbour AChR protein. Both bulk and shell lipids in the membrane are in the liquid-ordered (l_o) phase, with decreasing polarity towards the protein molecule [152]. That is, the protein-vicinal lipid-belt region is more ordered than the bulk, bilayer lipid, and yet the two lipid regions form a continuum from the physico-chemical point of view. The protein-vicinal first-shell lipid is relatively immobilized with respect to motions both around and perpendicular to the long molecular axes of the lipid molecules, respectively, with rotational correlation times about 50–100 times longer than is typically found with fluid bilayer lipid [78,110,112,116,150,156]. Notice that even the first-shell lipid is quite distant from the ion pore in the receptor protein, as made apparent in the vertically sectioned AChR molecule depicted on the right. Yet mutations in the outermost ring, the TM4 region [108] have a profound influence on AChR ion channel function [109].

Electron microscopy experiments over 20 years ago already indicated that about half the mass of the AChR protein protrudes into the extracellular space, about 30% corresponds to TM domains, and the remainder is in the cytoplasmic compartment [125–128]. The agonist recognition domains of the AChR were also located in the extracellular portion of the macromolecule (ref. [126], and see review in [129]) at a distance of about 25 Å from the apex of the AChR [130] and about 30 Å from the membrane surface [131–133]. The search for functionally relevant regions of the protein based on this «topographical anatomy» of the AChR protein was thus targeted to the TM domains.

Site-directed mutagenesis of the AChR combined with patch-clamp electrophysiological and photoaffinity labeling experiments with noncompetitive channel blockers support the notion that one of the TM AChR regions, the M2 domain, lines the walls of the pore. The data are also indicative of α-helical periodicity in the residues exposed to the lumen of the AChR channel [134]. NMR studies of the M2 segment of the δ subunit indicate that this domain is inserted in the bilayer at an angle of 12° relative to the membrane normal, in a totally α-helical configuration [135]. Analogously, a synthetic peptide corresponding to the *Torpedo* αM2 segment in organic solvents also adopts a totally α-helical configuration [136]. Cryoelectron microscope data confirmed that M2 forms the innermost ring of membrane-spanning segments, isolated from membrane lipids [137,138].

During the 1990s, cryoelectron microscopy fell short of revealing the structure of the other putative TM domains (M1, M3 and M4). In fact, a large portion of this AChR region was postulated to be arranged in the form of a β-barrel outside the central rim of M2 channel-forming rods [139]. This interpretation contrasted with photoaffinity labeling studies, in which the observed periodicity of the lipid-exposed residues in M4 and M3 was consistent with an α-helical pattern [140–142], and with deuterium-exchange Fourier transform infrared spectroscopy studies indicating a predominantly α-helical structure in the AChR TM region [143]. In addition, secondary structure analysis (CD and Fourier transform infrared spectroscopy) of isolated and lipid-reconstituted TM AChR peptides indicated α-helical structure for M2, M3, and M4 segments [144]. Furthermore, a synthetic peptide corresponding to the αM3 segment of *Torpedo* AChR exhibited a totally α-helical structure by two-dimensional ^{1}H-NMR spectroscopy [145]; NMR studies of a synthetic γM4 peptide are also compatible with an α-helical secondary structure [146].

Considerable advance in defining the structure of the AChR at atomic resolution [147] resulted from crystallographic studies of a water-soluble ACh-binding protein from a snail. The structure of this protein, highly homologous to the water-soluble extracellular domain of the AChR protein proper [147], provided the first truly high-resolution data of the region of the AChR involved in agonist recognition, the first step in the cascade leading to channel opening. More recent work has resulted in the crystallization of the actual water-soluble extracellular domain of the mouse AChR α-subunit bound to the competitive cholinergic antagonist α-bungarotoxin. The crystal structure was solved at 1.94 Å resolution [148].

6.2. The lipid shell surrounding the AChR outer ring is different from the bulk lipid in lipid contents and dynamics

The cryo-electron microscopy data of Unwin and coworkers [137–139] at 4 Å resolution provided inspiring insights into the structure of the AChR and particularly relevant to the subject of this review, the electron microscopy data revealed interesting features

of the membrane-embedded domains of the AChR protein. The occurrence of three concentric rings in the AChR region has been described [108]: (a) an inner ring exclusively formed by five M2 segments, corresponding to the walls of the AChR ion pore, which have no contact with the membrane lipid; (b) a middle ring, formed by 10 helices corresponding to the M1 and M3 TM segments. This middle ring is separated from the inner five-member ring of M2s, and its outer face is exposed to lipids and also to (c) the outermost ring, consisting of five M4 segments.

6.2.1. Composition and relative affinities in the lipid shell

The FRET studies using the SM fluorescent derivative also yielded information on the relative affinity of the SM derivative for the AChR protein. Thus, Py-SM exhibited a moderate-to-low selectivity for the protein-vicinal lipid domain, with a calculated relative affinity Kr@0.55 [149]. This figure should be added to the list of known selectivities of other lipids for the AChR calculated from early ESR experiments: PS (0.7), PC (1.0), PE (1.1), PA (2.7) and stearic acid (4.1) by Ellena and coworkers [114], and to those determined by us more recently, also using ESR techniques [150], allowing a classification of lipids according to their selectivity for the AChR protein. The study by Mantipragada et al. [150] provided the first detailed description of the dynamic composition of "first-shell" lipids in the belt region surrounding a receptor protein. AChR-vicinal lipids fall into three categories: (a) a high specificity group, constituted by fatty acids like stearic acid, cardiolipin and phosphatidylinositols [150–151], androstanol [116–151], and phosphatidic acid [114]; (b) an intermediate group, made up of SM [149], PS and PG [150], and (c) a moderate-to-low specificity group, where we find PC, PE, and the gangliosides GD1b, GM1, GM2 and GM3 [150].

It is interesting to note that owing to the high-packing density of AChR molecules in the postsynaptic membrane, only three to four phospholipid layers of "bulk" lipid separate the protein-vicinal first layer from the nearest-neighbour first-layer lipid surrounding an adjacent AChR protein [109]. In *Torpedo* receptor-rich native membranes prepared from electrocytes, *all* lipids in the membrane are in the liquid-ordered (l_o) phase, with decreasing polarity towards the AChR protein molecules [152]. This is because the protein-vicinal lipid-belt region is more rigid and ordered than the bulk bilayer lipid, as determined by Laurdan GP measurements using FRET [152,153]. However, the receptor-vicinal and the bulk lipid regions form a single, liquid-ordered phase from the physico-chemical point of view.

6.2.2. Dynamics of the lipid shell

The mobility at the lipid shell surrounding the AChR protein (the AChR-vicinal lipid, see Fig. 8) is reduced relative to that of the bulk membrane lipid, giving rise to a two-component ESR spectrum from which the number and selectivity of the lipids at the lipid–protein interface may be quantitated (see e.g. [116]). The protein-induced restrictions on the mobility of lipids exhibits selectivity. Spin-labeled fatty acids and anthracene [118] phospholipids [116], steroids and phosphatidic acid (PA) exhibit higher selectivity than other kinds of lipid [114,119]. This permitted the formulation of a hypothesis on the possible functional implications of the immobilization and the inherent topographical relationship (see Fig. 8). The crucial relationship between lipid and AChR protein became apparent in ion-flux studies showing that cholesterol and negatively charged phospholipids were required to support the gating activity of the AChR [121,122,154] whereas fatty acids blocked the ion-flux response [155]. The latter was interpreted as the perturbation of the interaction between AChR and cholesterol/negatively charged phospholipids.

How 'rigid' is the lipid surrounding the AChR protein relative to the fluid bilayer ('bulk') lipid? The protein-vicinal lipid is relatively immobilized with respect to motions both around and perpendicular to the long molecular axes of the lipid molecules, i.e., with rotational correlation times ~50–100 times longer than is typically found with fluid bilayer lipid [110]. The protein-vicinal lipid also exhibits a lower degree of penetration of water molecules, thus rendering it less polar than the bulk bilayer lipid [152,153]. Another dynamic aspect that characterizes the two lipid regions is the relatively high exchange between the two moieties: although the AChR-vicinal lipid is expected to have a lateral diffusion coefficient 50–100 times slower than that of the fluid bilayer lipid, i.e. $\sim 10^5$ s^{-1} [110], the lipid exchange process between the AChR-vicinal lipid and the bulk lipid exhibits rates in the order of $1–5 \times 10^8$ s^{-1}. Lipid species displaying selectivity for the AChR protein (see previous section) spend longer on average in the immediate vicinity of the protein; hence they are concentrated relative to those lipids exhibiting little or no selectivity. In systems where the selectivity of the lipid for the protein is changed by varying the pH or the ionic strength, it has been shown that the on-rate remains constant, whereas the off-rates reflect the specificity of a given lipid, which is independent of the lipid–protein ratio [156] (see also Fig. 8).

6.3. Cholesterol, sphingomyelins and the AChR

Here we shall discuss some structural aspects of the cholesterol–AChR interactions. The reader is referred to a recent topical review for the treatment of the functional modulation exerted by cholesterol on the ion channel properties and on the endocytic and exocytic trafficking of the receptor protein [73].

Early studies reported that cholesterol increased the α-helix content of the AChR. The sterol was postulated to stabilize AChR structure by packing its rigid planar ring into grooves of transmembrane helices [157], as is further discussed in other sections of this review. Circular dichroism spectroscopy [158], Raman spectroscopy [159] and $^1H/^3H$ exchange studies [160] detected no great differences in structure or solvent accessibility between the two main conformational states of the AChR, i.e., that occurring in the absence of agonist (the "resting" state) and the thermodynamically preferred conformation observed upon prolonged exposure to the agonist (the "desensitized" state), respectively. The accessibility of AChR fluorophores to membrane probes between resting and desensitized forms of the AChR is also different [161], as is the accessibility of residues near the ligand recognition site [162] and the transmembrane domains [163]. Castresana et al. [164] reported that the helical content of the AChR was not affected by addition of agonist, whereas the proportion of β-structure decreased to 24% concomitant with an increase in unordered structure. Lack of cholesterol in an asolectin reconstitution system produced an increase of disordered structure in *Torpedo marmorata* AChR [165]. Addition of exogenous cholesterol resulted in restoration of the proportion of ordered structures in asolectin liposomes but not in liposomes prepared from egg phosphatidylcholines, leading these authors to suggest that a component other than PC is needed for the restoration of AChR structure in the presence of cholesterol. Using Fourier transform infrared spectroscopy, Méthot et al. [166] found 39% α-helix, 35% β-sheet, 20% random coil and 6% turn in *Torpedo californica* AChR reconstituted in dioleoyl-phosphatidylcholine, dioleoyl-phosphatidic acid and cholesterol (3:1:1). In another series of studies also using Fourier transform infrared spectroscopy, Görne-Tschelnokow et al. [167] concluded from their data that the transmembrane region of the AChR contains 40% β-sheet plus turn structure. With the same technique, Baezinger and Méthot [168] produced experimental evidence supporting an all-helical model of the AChR transmembrane region. Thus, the relative proportions of helical structure found in these studies were in general agreement with that expected for the 25 AChR helices embedded in the membrane bilayer; that is, the experimental data reveal that the AChR exhibits sufficient α-helical content to account for four-helical transmembrane segments in each subunit and still provide some helical content to the extracellular region.

In terms of the dynamics of the structure, the Fourier transform infrared spectroscopy studies of Baezinger's group [168,169] indicated that $^{1}H/^{2}H$ exchange kinetics was slower in the presence of dioleoyl-phosphatidic acid or cholesterol, suggesting that the lipid environment modulates the conformational dynamics of the membrane-embedded peptide hydrogens that exchange with deuterium. No great changes in secondary structure were observed in the presence of cholesterol also using Fourier transform infrared spectroscopy [170]. An early study using this technique in combination with radioactive ion-flux experiments [171] suggested that lipid mixtures containing saturated phosphatidylcholine, anionic phospholipid (phosphatidic acid) and cholesterol stabilize the receptor in the resting state and allow agonist-induced state transitions. All in all, the wealth of evidence points to the need for cholesterol in the membrane milieu of the AChR for maintaining its correct structure and the ability to undergo conformational transitions.

The other class of lipid that is present in ordered domains in the membranes is that of sphingolipids, which account for about 5% of the total lipid content of AChR-rich membranes obtained from the electrocytes of Torpedinidae fish [172,173]. The topography of sphingomyelin (SM) in the *Torpedo* AChR-rich membrane has been the subject of cytochemical and biophysical studies [149]. The affinity of SM for the AChR protein has been established using fluorescence techniques: the lipid exhibits only moderate-to-low affinity for the receptor protein [149]. More recently, a role for SM in AChR trafficking has been disclosed; biochemical and fluorescence microscopy studies showed that inhibition of sphingolipid biosynthesis resulted in the accumulation of unassembled AChR oligomers in the endoplasmic reticulum. These experimental observations led to the suggestion that sphingomyelins could play a "chaperone-like" effect on the AChR biosynthetic pathway, affecting both the efficiency of the assembly process and subsequent receptor trafficking to the cell surface [70].

7. Conformational effects of sphingolipids: from physical chemistry to physiology

7.1. The sphingolipid binding domain

The proposal that protein–glycolipid interactions follow a universal scheme based on a unique biochemical mechanism has emerged from a comprehensive molecular modelling approach combined with thorough physicochemical studies of peptide–glycolipid and/or protein–glycolipid interactions [174–176]. This is the concept of the glycolipid binding domain, or, more generally, of the sphingolipid binding domain (SBD), which has been extensively reviewed [2,5,177–179]. Briefly, the domain generally consists of a hairpin loop with specific amino acid requirements. Aromatic residues stack onto the sugars rings of GSLs (CH–Pi stacking), whereas charged residues can form electrostatic bonds with sphingomyelin. An SBD displaying a single aromatic residue, but no charged residue, can efficiently bind to neutral GSLs [180]. The simultaneous presence of aromatic and basic residues extends the GSL repertory to gangliosides [181,182]. Finally, charged residues (either acidic or basic) are commonly found in synthetic peptides that bind to sphingomyelin [175,183].

In the case of membrane proteins, SBD are generally located in extracellular regions close to the polar–apolar interface of the membrane, so that the side chains of the amino acid residues of the domain can bind to the polar headgroup of the sphingolipid [5]. Indeed, in the three-dimensional structure of the nicotinic acetylcholine receptor alpha chain, an SBD can be predicted at the periphery of the subunit, in contact with membrane lipids (Fig. 9A). This SBD contains two aromatic residues whose side chains are oriented toward the interior of the loop and contribute to its stabilization. In contrast, two Glu and one Lys residues, fully accessible for membrane lipids, can theoretically interact with sphingomyelin. This domain could be involved in the regulation of the conformation and trafficking of the AChR [70]. For comparison, we also showed the structure of the first SBD ever characterized, i.e., the V3 loop of the HIV-1 surface envelope glycoprotein gp120 (Fig. 9B). This SBD recognizes both GalCer and sphingomyelin [2] which is consistent with the accessibility of Phe and Arg side chains.

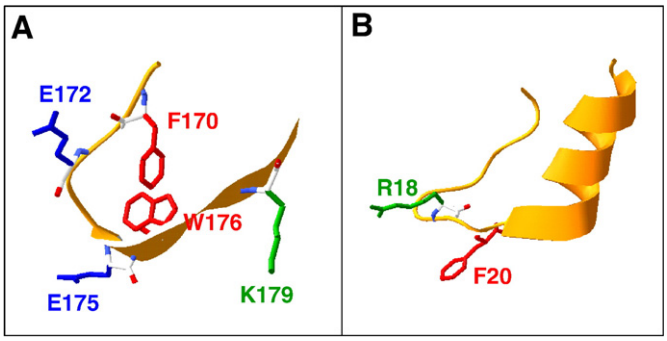

Fig. 9. Examples of sphingolipid binding domains. (A) SBD in the alpha chain of the acetylcholine receptor (chain A) retrieved from the Protein DataBank (PDB) entry 2BG9 [131]. This domain is located at the boundary between the extracellular (EC) and transmembrane (TM) regions of the receptor shown in Fig. 7. The aromatic residues (F170 and W176) are buried in the three-dimensional structure of the receptor. The charged residues (E172, E175, and K179) are located on the protein/membrane interface, consistent with an interaction with surrounding membrane lipids, especially sphingomyelin. (B) The prototype SBD (and the first ever characterized): the V3 loop of HIV-1 gp120. In this case, both the aromatic F20 and the basic R18 residues are accessible to sphingolipids (sphingomyelin and GalCer).

Functional sphingolipid and/or glycolipid binding domains have been identified in a wide range of proteins, including Fas/CD95, class I MHC, EGF and PDGF receptor, Thy-1, prominin (CD133), intestinal alkaline phosphatase, the glucose transporter GLUT-1, *Helicobacter pylori* adhesin, shiga and botulinum toxins, hepatitis C virus non structural protein, as well as various amyloid proteins [2,5,177,179–181,183–184]. All these SBD have been successfully predicted by either sequence or structure similarity searches using the HIV-1 gp120 V3 loop as template. Finally, a fluorescent probe based on the SBD identified in the beta-amyloid peptide [175] has been shown to specifically interact with sphingolipids and cholesterol-dependent raft domains on live neural cells, further demonstrating the robustness of the SBD concept [185].

7.2. Molecular mechanisms: CH–Pi interactions and GSL-associated chaperone activity

As discussed above, it has been shown that sphingolipids are necessary for the export of the AChR in the early secretory pathway [70]. In this case, it is likely that the sphingolipids (especially sphingomyelin) have a chaperone effect at early stages of the acetylcholine receptor biosynthetic pathway. A similar effect of sphingolipids has been demonstrated in the case of the cellular isoform of the prion protein [186], which also contains a sphingolipid binding domain [175,179].

What are the molecular mechanisms behind the chaperone activity of sphingolipids? Though this issue has not yet been fully resolved, some interesting progress has been made by studying the molecular mechanisms controlling the interaction of sugars with aromatic compounds [83]. In solution, an aromatic ring tends to stack onto another aromatic ring to avoid the thermodynamically unfavourable contacts with water molecules. The resulting Pi–Pi stacking interaction contributes to major biological functions, such as the stability of the DNA double helix and protein folding [187]. However, it has recently been shown that the self-aggregation of adenine in water solution, which results from a Pi–Pi stacking-driven process, can be abrogated by free sugars, the most active being beta-galactose [83]. Beta-galactose is unique among hexoses because its pyranic

cycle has two distinct surfaces, one apolar and the other polar [83,176]. The apolar surface of galactose provides a perfect complementary surface for the aromatic cycle, onto which it readily binds through a CH–Pi stacking interaction, with the CH groups of galactose facing the delocalized Pi electronic cloud of the aromatic ring as shown in Fig. 10 for toluene [176] and phenylalanine. In both cases, water molecules are excluded from the space between the stacked cycles and rejected at the periphery of the complex. This entropic-driven process further stabilizes the sugar-aromatic complex.

Now, what happens if we extrapolate this physical chemistry property of sugar-aromatic systems to GSL–protein duets? The side chains of aromatic amino acids tend to be protected from the solvent so that they are generally buried in the three-dimensional structure of the protein. This feature is so frequent that when the aromatic side chain of a Trp residue is exposed on the surface of a protein, it generally indicates a ligand binding site [188]. By providing a complementary surface for accessible aromatic side chains, the glycone part of membrane GSLs may interfere with the natural propensity of aromatic cycles to bury themselves in apolar crevices of the protein. By doing this, the GSL induces a significant conformational rearrangement which can be considered as a chaperone-like effect [189]. From a physical chemistry point of view, the intramolecular Pi–Pi stacking interactions between aromatic residues of the protein are substituted by intermolecular (GSL–protein) CH–Pi stacking interactions. This 'stacking exchange' between Pi–Pi and CH–Pi is possible at minimal energy cost because these interactions share the same geometry [177]. The consequence is a conformational effect which may, in the specific case of a neurotransmitter receptor, affect the ligand binding pocket and/or the coupling machinery to the effector functions (protein G or channel activation). Conceptually, similar effects could be obtained through electrostatic interactions between proteins and charged sphingolipids such as sphingomyelin [70,175].

7.3. Fine tuning of GSL conformation by cholesterol

Basically, the binding of cholesterol to the transmembrane helices of receptors could also produce important conformational effects on these proteins [81]. This certainly contributes to the regulatory effects cholesterol exerts on many receptors [71–73,96]. The molecular mechanisms involved in the association of cholesterol with transmembrane helices have not been fully deciphered, but aromatic residues seem to play an important role (Fig. 6). Indeed, aromatic cycles have the potency to stack onto the rigid cycles of cholesterol, thereby forming stabilizing CH–Pi interactions. In addition, the methyl groups of the beta side of cholesterol (Fig. 2) can insert into local crevices of the transmembrane helices which are delineated by the branched side chains of aliphatic amino acid residues such as the frequently encountered Ile and Val. This suggests that, if the beta face of cholesterol interacts with a transmembrane helix, its smooth alpha side can interact with a sphingolipid (Fig. 11). The possibility therefore exists of forming a ternary complex in which cholesterol interacts simultaneously with one of the transmembrane domains of the receptor and with a GSL. The glycone part of the GSL protrudes from the membrane and its orientation is largely determined by the OH group of cholesterol [5]. In other words, cholesterol can simultaneously control the conformation of the GSL and the conformation of the receptor. If one considers that the GSL itself can bind to the receptor and affect its conformation, it becomes evident that we are facing a complex network of molecular crosstalk between

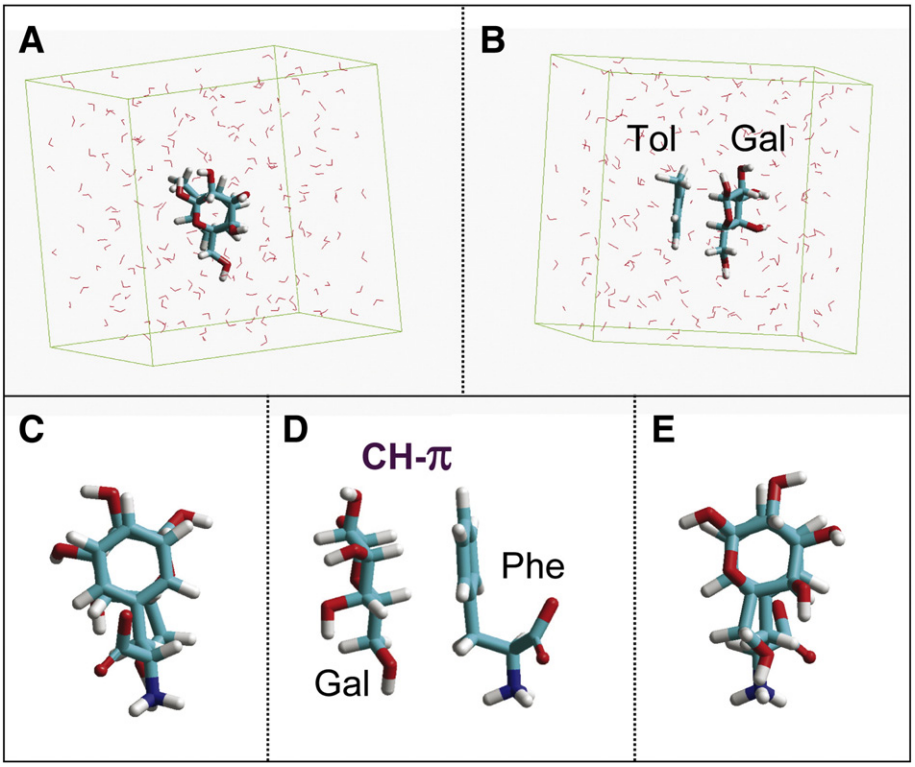

Fig. 10. The CH–Pi stacking interaction. Upper panels: Molecular modelling of a toluene–galactose complex in water (modified from ref. [176]). The geometry of a molecular complex between toluene (Tol) and β-D-galactose (Gal) was first minimized with the Polak-Ribiere algorithm. The molecules were then introduced in a periodic box of 18.7 $Å^3$ in the presence of 216 water molecules. Molecular dynamics simulations with the MM+ force field were then conducted for 10 ps with Hyperchem 7 software. Two distinct views of the complex (A and B) are shown, along with the 216 water molecules. Lower panels: Molecular modelling of galactose–phenylalanine (Gal–Phe) interactions in water under three distinct orientations, viewed from Phe (C), profile (D) and from Gal (E). Note the near-perfect geometric superposition of both cycles forming the CH–Pi stacking interaction. The molecular modelling simulations were performed using the same conditions as for the toluene–galactose complex. The surrounding water molecules were not shown to improve the clarity of the models.

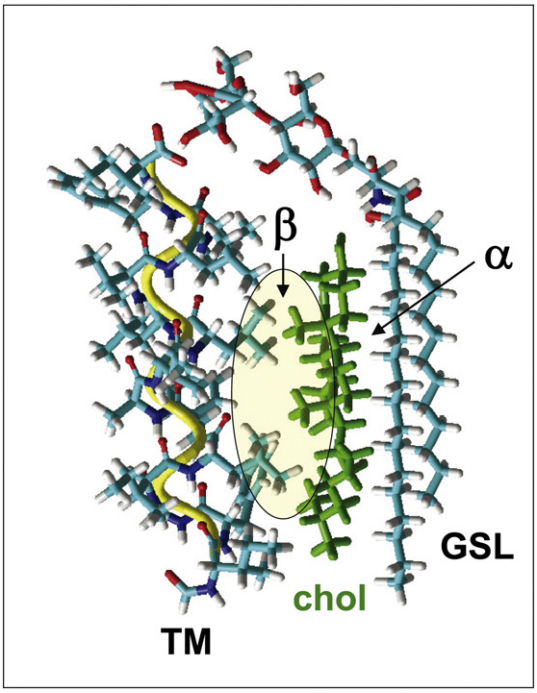

Fig. 11. Ternary complex beween a transmembrane helix, cholesterol and a GSL. Cholesterol (chol) is in green, with its smooth alpha face interacting with the ceramide part of the GSL (on the right). A representative transmembrane domain (TM) with Ile and Val side chains interacts with the beta face of cholesterol (yellow surface). Note how the polar head of the GSL has adapted its shape to the TM–cholesterol complex. This indicates that each partner of the complex might influence the conformation of the others.

the receptor and its surrounding lipids. The multiple possibilities offered by this molecular interplay would allow a fine tuning of receptor trafficking, sensitivity to neurotransmitters, and functional coupling to effector mechanisms. Deciphering this molecular language will be a hard but fascinating task in the coming years.

8. Conclusions and perspectives

Several lessons can be drawn from this overview. Starting from a list of clearly identified biochemical mechanisms involving specific lipid–protein interactions, we have attempted to shed some light on their physiological significance for neurotransmitter receptor function with special emphasis on serotonin and acetylcholine receptors, which include two main types of receptors: G protein-coupled proteins with seven transmembrane domains, and multimeric assemblies of protein subunits forming a ligand-gated ionic channel. Like all other transmembrane proteins, these receptors are surrounded by a shell of lipids which may primarily affect their trafficking between raft and non-raft fractions of the plasma membrane. In addition, these receptors can specifically interact with cholesterol and sphingolipids through typical binding sites located in both the transmembrane helices and the extracellular loops. These lipidic ligands modify the conformation of the receptor, affecting both neurotransmitter binding and signal transducing functions.

Biochemical fact 1: Neurotransmitters bind to GSLs.

Physiological consequence: Following release from synaptic vesicles, neurotransmitters are attracted by GSLs (chiefly gangliosides) in the post-synaptic membrane. This has two main consequences: (i) concentration of neurotransmitter monomers in GSL-enriched areas of the membrane, where neurotransmitter receptors are also concentrated; (ii) GSL-assisted delivery of the neurotransmitter to its receptor, according to the two-receptor model developed by C. Montecucco for neural toxins [47,48]. In some instances, the GSL could affect the conformation of the neurotransmitter, thereby facilitating the preselection a receptor subtype. This could be particularly important for serotonin, which can adopt distinct conformations and interacts with several distinct receptors.

Biochemical fact 2: Some neurotransmitter receptors are associated with lipid domains.

Physiological consequence: Although not formally demonstrated for all neurotransmitter receptors, the available data strongly suggest that lipid domains are indeed the regions of the plasma membrane where some receptors act [190]. This is the case for G protein-coupled receptors which, upon binding of the neurotransmitter, interact with and activate G proteins that are themselves present in lipid domains. In this respect, lipid domains allow the functional coupling between the two leaflets of the plasma membrane, the extracellular one which captures the messengers, and the intracellular one which triggers G protein action. The other reason is that cholesterol and sphingolipids, which exert important regulatory effects on these receptors, are typical raft lipids. Cholesterol and sphingolipids might be particularly important for ligand-gated ionic channels whose subunits have to assemble and cooperate before and upon neurotransmitter binding (see below).

Biochemical fact 3: Cholesterol binds to some neurotransmitter receptors.

Physiological consequence: The transmembrane domains of neurotransmitter receptors have a major role in both the acquisition and dynamics of the active three-dimensional structure of the receptors. In the case of G protein-coupled serotonin receptors, the binding pocket is located inside the membrane, mainly between the 2nd and 3rd transmembrane helices. A cholesterol binding motif with conserved amino acid residues has been characterized in the vicinity of the ligand binding pocket. Correspondingly, cholesterol has a major effect on ligand binding. Moreover, the transmembrane domains are those that link the ligand binding pocket to the switch responsible for signal transduction triggering, so cholesterol can also directly control this process. Cholesterol binding sites have not been fully identified in the case of the AChR, but ligand binding, stability at the membrane and trafficking to the cell surface are all dependent on normal cholesterol levels in the cell. Finally, one should be aware that cholesterol is a bifacial molecule with two topologically distinct surfaces, one rough and the other smooth. This allows cholesterol to interact at the same time with a transmembrane domain of the receptor and with a GSL, thereby inducing conformational adjustments on both partners (the GSL and the receptor).

Biochemical fact 4: Sphingolipids bind to neurotransmitter receptors.

Physiological consequence: This is perhaps the main mechanism of regulation of receptor function by membrane lipids, and certainly one essential function of brain sphingolipids. The extracellular regions of the receptors can display structurally related but functionally specific SBDs for neutral, cationic, and/or acid sphingolipids. These sphingolipids induce profound conformational changes in these receptors, which can affect both ligand binding and signalling pathways. The molecular mechanisms involved in this chaperone effect of sphingolipids has remained elusive for many years, but recent data obtained from physical chemistry studies have emphasized the importance of CH–Pi stacking interaction between GSLs and aromatic side chains of proteins. The conformational effects of sphingolipids are generally cholesterol-dependent, because cholesterol binds to most sphingolipids and largely determines their biologically active conformation.

In summary, several decades of research have been necessary to untangle the skein of a complex network of molecular interactions between neurotransmitters, their protein receptors, cholesterol and sphingolipids. Such sophisticated crosstalk between all four distinctive partners may allow a fine biochemical tuning of synaptic transmission. We surmise that this aspect of synaptic regulation,

involving sphingolipid/cholesterol regulation of neurotransmitter receptor function, will lead to challenging discoveries in the neurosciences.

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